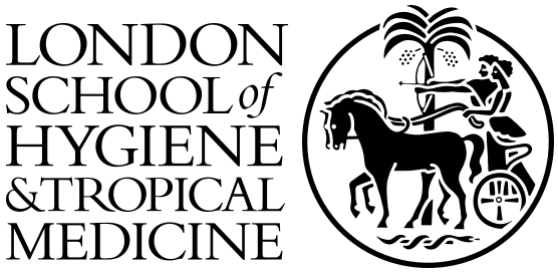

Manuscript type: Research article

Running title: Sputum decontamination for mycobacteria

Title: Evaluation of OMNIgene® SPUTUM reagent for mycobacterial culture

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Summary

Setting: National Mycobacterium Reference Laboratory, Borstel Germany.

Objective: To evaluate the effectiveness of OMNIgene® SPUTUM reagent in comparison to a method using N-acetyl cysteine (NALC)-NaOH with regards to mycobacterial recovery and contamination of broth and solid cultures.

Design: Sputum samples from patients with tuberculosis and other respiratory diseases underwent decontamination with either NALC-NaOH-based (MycoDDR™) or OMNIgene® SPUTUM reagent. The decontamination procedure was assigned by block randomization. Samples were inoculated on Löwenstein-Jensen, Stonebrink and mycobacterial growth indicator tubes (MGIT). Mycobacterial recovery from samples spiked with *Mycobacterium tuberculosis* following decontamination was determined.

Results: Eighty-five samples were randomized to NALC-NaOH and 84 to OMNIgene® SPUTUM reagent. Mycobacterial recovery was significantly lower for samples processed with OMNIgene® SPUTUM reagent vs. the NALC-NaOH method across all media types. Culture contamination was lower with NALC-NaOH reagent 9.4-12.9% vs. 28.6-29.8% on solid media. No growth was observed in MGIT among samples spiked with 10,600-16,800 CFU of *M. tuberculosis* following decontamination with OMNIgene® SPUTUM reagent.

Conclusion: Low mycobacterial recovery, especially in MGIT, observed in this study suggests that OMNIgene® SPUTUM reagent might not be compatible with the MGIT system. More extensive field evaluations of the OMNIgene® SPUTUM reagent are warranted to demonstrate a significant benefit over current methods.

Introduction

Worldwide, an estimated 10.4 million new tuberculosis cases occurred in 2016; with more than a third of these cases remaining undiagnosed.[1] Rapid and accurate diagnosis of tuberculosis is critical for timely initiation of treatment and, ultimately, tuberculosis control.

New molecular diagnostics with sensitivities of 70-90% to detect *Mycobacterium tuberculosis* complex DNA in primary samples have changed the diagnostic landscape.[2, 3] However, culture, using both broth and solid media, remains the standard for establishing the laboratory-based diagnosis of tuberculosis.

The mycobacterial cell wall with its thick and relatively impermeable layer of mycolic acid has unique characteristics. The complexity of the cell wall represents a challenge to the organism, requiring specialized mechanisms to allow cell division to occur and resulting in prolonged generation times compared to gram-positive or -negative bacteria.[4] Respiratory samples are therefore specially treated, a process called ‘decontamination’ before inoculation and incubation of broth and solid media. Decontamination and the supplementation of broth and media with antibiotics and antifungals are intended to prevent overgrowth of the slower growing mycobacteria by concomitant flora of specimens from non-sterile sites. Decontamination procedures aim at decreasing viability of gram-positive and -negative bacteria and fungi while interfering as little as possible with mycobacterial viability and growth.[5]

Given the prolonged generation times of mycobacteria compared to other bacteria and fungi, prolonged transport at ambient temperature may result in overgrowth of contaminants even before samples have been processed.[6, 7] OMNIgene® SPUTUM reagent (DNA Genotek, Ottawa, Canada) has been specifically developed to optimise the pre-analytic phase. It is a transport reagent that liquefies and decontaminates sputum while preserving viability of *M. tuberculosis*

for up to 8 days at temperatures as high as 40ºC. According to the product information sheet, OMNIgene® SPUTUM reagent added to sputum samples is directly compatible with all molecular tests and gold standard tuberculosis tests including smear microscopy and liquid culture (BD BACTEC™ MGIT™ 960 System, BD, Franklin Lakes, NJ, USA) and the Xpert® MTB/RIF assay (Cepheid, Sunnyvale, CA, USA).[8, 9]

This study aimed to evaluate the effectiveness of OMNIgene® SPUTUM reagent with regards to mycobacterial recovery and contamination of broth and solid cultures versus a commercially available CE-marked N-acetyl cysteine (NALC)-NaOH method using sputum samples submitted by patients treated for tuberculosis or admitted for the management of other pulmonary diseases.

Methods:

Sputum samples

Over a period of 10 weeks (August-October 2016) patients admitted to the chest hospital in Borstel submitted sputum samples on two days of the week; samples were processed on the day of collection by the National Mycobacterium Reference Laboratory. The method used for decontamination was assigned by block randomization with the unit of randomization being the day of the week.

Decontamination procedures

Samples were either processed using a CE-marked NALC-NaOH method (MycoDDR™, IMMY, Norman, USA)[10] or OMNIgene® SPUTUM reagent (DNA Genotek, Ottawa, Canada)[9] according to the manufacturer's instructions at the time of the study. In brief for the NALC-NaOH method equal volumes of detergent (2.5% NaOH reagent) were added 1:1 to samples

measuring up to 10 ml, samples were split if the volume was >10ml. Samples were incubated for 15 min at room temperature and vortexed every 5 min for 30 sec. Kit-specific neutralisation buffer was added until a colour change indicated a neutral pH, followed by 15 min centrifugation at 3000 x g at 8°C. Supernatants were discarded and pellets re-suspended in kit-specific resuspension buffer to achieve a final volume of 2 ml. The OMNIgene® SPUTUM reagent was added in equal volume to each sample. Samples were incubated for 20 min at room temperature with intermittent vortexing every 5 min, followed by 15 min centrifugation at 3800 g at room temperature. Supernatants were removed and pellets suspended in sterile water aiming for a final volume of 2 ml. Before inoculation pH-values were measured using a pH test paper (ChemoLine GmbH, Hennef, Germany).

Mycobacterial culture

Following decontamination 100 µl of the resuspended pellet were inoculated on Löwenstein-Jensen (LJ) and Stonebrink slants (Artelt-ENCLIT GmbH, Wyhra, Germany) supplemented with antimicrobial drugs (polymyxin B, amphotericin B, carbenicillin, and trimethoprim [PACT]). In addition, 500 µl of the resuspended pellet were inoculated in mycobacterial growth indicator tubes (MGIT, Becton Dickinson, Heidelberg, Germany) supplemented with an antimicrobial mixture containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) and incubated at 37º for a maximum of 42 days. Solid media were reviewed weekly for growth of mycobacteria for a total of 8 weeks. Contamination and mycobacterial recovery rates were calculated stratified by media and decontamination method. In addition, time to positivity in days was determined for broth (MGIT). For positive MGITs an aliquot of 1 ml was taken from the bottom of the tube, centrifuged for 10 min at 3600 x *g* and the supernatant

discarded. 10 µl of the pellet were used to prepare a microscopy slide. Following heat fixation, Kinyoun staining was performed to determine the presence of acid fast bacilli or other bacteria. The remaining pellet was resupended in 500 µl of NaCl, 50 µl were inoculated on Columbia blood agar (Beckton Dickinson, Heidelberg, Germany) and incubated at 37 C for 24 hours. A positive MGIT was classified as contaminated if no acid-fast bacilli were seen on microscopy and other non-acid-fast bacteria were present on microscopy and/or on blood agar. Identification of mycobacteria was performed using the Genotype HAIN CM or TBC (HAIN Lifescience, Nehren, Germany) as per the manufacturers' instructions.

Spiked samples

A suspension of 1.5% methylcellulose (Sigma-Aldrich, Germany) was spiked with *M. tuberculosis* H37Rv aiming for a final concentration of 10,000-20,000 colony-forming units (CFU)/ml. 100 µl of the undiluted suspension and serial dilutions (1 in 10, and 1 in 100) were inoculated in

7H10 agar plates in duplicates and incubated at 37ºC for 4 weeks. CFU were determined by calculating the mean of all six 7H10 agar plates taking the dilution into account.

To determine the effect of NALC-NaOH decontamination and OMNIgene® SPUTUM reagent on viability of mycobacteria 2 ml of the bacterial suspension underwent processing with NALC-NAOH or OMNIgene® SPUTUM reagent prior to inoculation. The bacterial suspension without any pre-treatment served as a growth control.

LJ and Stonebrink slopes were inoculated with 100 µl, while MGIT were inoculated with 500 µl resuspended sediment (or the untreated bacterial suspension). Samples were set up in duplicate

and experiments were repeated on two different days (experiment I and II). Cultures on solid media were checked weekly for mycobacterial growth.

Statistical analysis

Collected data were entered in an Excel database (Microsoft Office). Statistical analysis was performed using Stata version 14 (Stata-Corp, TX, USA). We compared the proportion of contaminated cultures using the NALC-NaOH method as a reference. The χ^2 was used to evaluate differences in proportions. The Wilcoxon Rank sum test was used to compare medians. The level of significance was set at $\alpha=0.05$.

Ethics

Individual patient consent was obtained. The study was approved by the Ethical Committee of the University of Lübeck, Germany (#16-209).

Results

A total of 169 samples from 34 patients were included in the study, 85 samples were randomized to NALC-NaOH and 84 to OMNIgene® SPUTUM reagent. Most samples were submitted by patients on treatment for drug susceptible tuberculosis (n=82) and multi-drug resistant tuberculosis (n=74). There was no difference in sample distribution across methods (figure 1, $p<0.01$).

The proportion of samples revealing growth of *M. tuberculosis* was significantly lower for samples processed using the OMNIgene® SPUTUM reagent compared to samples processed with the NALC-NaOH method across all media (table 1). In the MGIT system only 6 (7.1%)

samples grew *M. tuberculosis* following treatment with OMNIgene® SPUTUM reagent compared to 17 (20%) when using the NALC-NaOH method. Median time to positivity was 9.5 days for the NALC-NaOH method and 13.9 days for OMNIgene® SPUTUM reagent. In the OMNIgene® SPUTUM reagent group more than a quarter of samples inoculated on LJ or Stonebrink were contaminated compared to 9.4-12.9% in the NALC-NaOH group. In contrast only 7.1% of samples processed using OMNIgene® SPUTUM reagent were contaminated in the MGIT compared to 20% of samples in the NALC-NaOH group. The median pH of the resuspended pellet was neutral (7) for samples treated with NALC-NaOH compared to 8.5 for samples undergoing the OMNIgene® SPUTUM reagent method.

Spiked samples had a mean of 16,800 CFU of *M tuberculosis* per ml in the first experiment and 10,600 CFU in the second experiment. The mean (average of the duplicate samples) time to culture positivity was 8.2 and 9.6 days for the growth control tubes and 11.5 and 12.0 days for suspensions treated with NALC-NaOH. No growth was recorded for suspensions treated with OMNIgene® SPUTUM reagent after 6 weeks of incubation. On solid media growth of *M. tuberculosis* was observed in samples undergoing either method (OMNIgene® SPUTUM reagent or NALC-NaOH), albeit growth was reduced for samples treated with OMNIgene® SPUTUM reagent.

Discussion

This study found that samples decontaminated using OMNIgene® SPUTUM reagent had lower mycobacterial recovery than those processed using the NALC-NaOH-based method.

Contamination rates on solid media were higher in samples treated with OMNIgene® SPUTUM reagent compared to NALC-NaOH.

According to the manufacturer, the OMNIgene® SPUTUM reagent is compatible with both solid media and MGIT. However, in this study we found significantly decreased mycobacterial recovery both on solid media and in MGIT when samples were processed using OMNIgene® SPUTUM reagent. For solid media, the increase in culture contamination might have directly impaired mycobacterial recovery due to overgrowth of the contaminant organisms. Time to culture positivity in MGIT appeared to be longer, although the number of positive samples was low. Furthermore, mycobacteria could not be recovered from broth using samples spiked with *M. tuberculosis*. Other studies have also found an increase in time to positivity when using OMNIgene® SPUTUM reagent in combination with the MGIT system.[11, 12] This might suggest that one or more of the components of the OMNIgene® SPUTUM reagent might interfere with components in the MGIT or directly with mycobacterial growth. While the exact components of OMNIgene® SPUTUM reagent are unknown, the product safety data sheet lists sodium dodecyl sulphate (SDS) 1-5%.[13] It is well established the SDS is not compatible with the MGIT system due to its strong binding to proteins present in the medium which can in turn impair mycobacterial growth (and possibly that of other bacteria).[14] This would result in both poor recovery and delayed time to detection, but also in relatively low "contamination rates". Another explanation of the low proportion of positive cultures across all media is the alkali pH following re-suspension. A high pH may cause injury or death to mycobacteria and delayed mycobacterial revival resulting in growth delays.[15]

The results of this study are in contrast with a study conducted in Nepal showing increased mycobacterial detection and decreased culture contamination in samples processed using the OMNIgene® SPUTUM reagent. However, cultures were performed on solid media only and incubation time of sputum samples with OMNIgene® SPUTUM reagent was prolonged (up to 8

161 days) rather than 20 min.[16] Length of incubation might influence performance of OMNIgene®
162 SPUTUM reagent. A study from Uganda reported lower proportions of positive cultures among
163 samples processed on the day of collection using OMNIgene® SPUTUM reagent compared to
164 NALC-NaOH, albeit the number of same-day processed samples was low.[12] However, across all
165 incubation durations, the number of samples revealing mycobacterial growth was lower in the
166 OMNIgene® SPUTUM reagent group compared to the standard of care.[12]
167 Recently, Genotek changed the OMNIgene® SPUTUM reagent sample processing instructions.
168 The minimum incubation time was increased from 20 to 30 min. The instructions now
169 recommend adding phosphate-buffered saline (PBS) to the mix of sputum samples and
170 OMNIgene® SPUTUM reagent before centrifugation. Generally longer incubation times might
171 be beneficial, while adding PBS might result in diluting some of the SDS, and the pH might get
172 closer to neutrality.
173 Our study did not show any benefit of OMNIgene® SPUTUM reagent over NALC-NaOH with
174 regards to contamination. This is in contrast with the findings from other studies reporting lower
175 culture contamination rates when using OMNIgene® SPUTUM reagent.[12, 16] The difference
176 might be explained by differences in patient populations. The majority of patients submitting
177 sputum samples for this study were on treatment for drug susceptible or resistant tuberculosis
178 and hence contamination rates were particularly high. The studies from Nepal and Uganda only
179 included patients who had symptoms suggestive of tuberculosis, but were not yet on treatment.
180 The strengths of this study are its cluster randomized design, the use of a challenging patient
181 population and the combined approach of using both patient samples and spiked samples. The
182 study is limited by its small sample size and the low number of positive samples.

In conclusion, this study suggests that OMNIgene® SPUTUM reagent might not be compatible with the MGIT system. This is supported by the lower proportion of positive cultures across solid culture and broth and the prolonged time to culture positivity. Rigorous field evaluations of the OMNIgene® SPUTUM reagent are needed to demonstrate a significant benefit over currently used methods.

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Conflict of interest: Kits for decontamination were provided free of charge from the manufacturers. None of the companies had any influence on the results of the study.

Table 1. Contamination and mycobacterial recovery

	Result	**NALC-NaOH MycoDDR™ N=85 N (%)**	**OMNIgene® N=84 N (%)**	**P value**
Löwenstein-Jensen	Growth of *M. tuberculosis*	18 (21.2%)	13 (15.5%)	0.006
	Contamination	8 (9.4%)	24 (28.6%)	
	No growth	59 (69.4%)	47 (56.0%)	
Stonebrink	Growth of *M. tuberculosis*	18 (21.2%)	14 (16.7%)	0.028
	Contamination	11 (12.9%)	25 (29.8%)	
	No growth	56 (67.9%)	45 (54.6%)	
Mycobacterial Growth Indicator Tubes	Growth of *M. tuberculosis*	17 (20.0%)	6 (7.1%)	0.001
	Contamination	17 (20%)	6 (7.1%)	
	No growth	51 (60.0%)	72 (85.7%)	
pH of the resuspended pellet, median (interquartile range)		7 (6.75; 7)	8.5 (8; 8.5)	0.001

Figure 1. Method used for decontamination according to diagnosis.

Each pair of bars represents an individual patient; black bars show samples processed using MycoDDR™ while light bars show samples processed using OMNIgene® SPUTUM reagent. DS= drug-susceptible; TB = tuberculosis, MDR = multidrug resistant

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